Electrophoretic and other Studies on Haem Pigments from *Rhodopseudomonas palustris*: Cytochrome 552 and Cytochromoid *c*

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1. Cytochrome 552 and cytochromoid *c* were extracted from *Rhodopseudomonas palustris* cells, purified and obtained in crystalline form. 2. Extinction ratios and amino acid compositions of the two pigments are reported. 3. When subjected to starch-gel electrophoresis in borate buffer, pH 8·8, each pigment migrated towards the cathode; oxidized cytochromoid *c* migrated more rapidly than its reduced form. 4. By a determination of electrophoretic mobilities in buffers of *I* 0·1 by using the moving-boundary method, the isoelectric point of cytochrome 552 was found to be at pH 10·6 and that of cytochromoid *c* at pH 9·7. 5. As obtained, cytochrome 552 was non-autoxidizable; cytochromoid *c* was autoxidizable but became considerably less so on alkaline treatment. 6. Discussion of the results includes a consideration of the isoelectric points of the pigments in terms of their amino acid composition.

When grown anaerobically in the light the purple photosynthetic bacteria contain appreciable concentrations of a *c*-type cytochrome and the related cytochromoid *c* (Kamen, 1963*a*; Porra & Lascelles, 1965). For this study, the two representative haemoproteins were prepared from *Rhodopseudomonas palustris* in crystalline form; as far as we know, the cytochromoid from this source has not previously been obtained as crystals.

During starch-gel electrophoresis at pH 8·8 both pigments migrated towards the cathode. Isoelectric points well on the alkaline side of neutrality are unusual, as most bacterial cytochromes have an isoelectric point less than 7 and all of the *c*-type cytochromes and cytochromoids from the photosynthetic bacteria so far examined have had isoelectric points less than 7 (Dus & Kamen, 1963). Further, Morita (1960), using a crystalline preparation of *Rps. palustris* cytochrome 552, has estimated the isoelectric point as 7·7, from a determination of electrophoretic mobilities by the moving-boundary method. In view of the well-known difficulties attendant on comparison of results from gel electrophoresis with those from the moving-boundary method it was decided to determine the electrophoretic mobilities of each pigment over a range of pH values in the Tiselius-type electrophoretic apparatus. To characterize the pigments by comparison with published information, spectrophotometric and other data obtained are also reported. de Klerk, Bartsch & Kamen (1965) reported a cathodic migration of the *Rps. palustris* haemoproteins cytochrome 552 and cytochromoid *c*, when these were subjected to starch-gel electrophoresis at pH 8·9.

A preliminary report of this work has been made (Henderson & Nankiville, 1965).

MATERIALS

Cultures. The strain of *Rps. palustris* used in this study was kindly provided by Dr M. D. Kamen, University of California, San Diego, Calif., U.S.A., and was originally strain no. 2137 from the collection of Dr C. B. van Niel, Stanford University, Calif., U.S.A. The organism was cultivated photosynthetically under aseptic conditions in what was essentially the medium used by Cohen-Bazire, Sistrom & Stanier (1957) for the cultivation of *Rhodospirillum rubrum*. The biotin used in the salt solution by these workers was replaced by 0·1% (w/v) Difco yeast extract; also, 0·1% (w/v) L-glutamic acid and 0·1% (w/v) sodium acetate trihydrate were used instead of the Difco casein hydrolysate. The cultures were grown at 34° in 12l. flasks equipped with cooling coils, under a bank of 300w tungsten lamps, the light-intensity at the culture surface being 600ft.-candles. Oxygen-free nitrogen was bubbled slowly through the cultures. The cells were harvested after they had reached the stationary phase (5–7 days) by use of a Cepa Schnell continuous-flow centrifuge (model LEI; Carl Padberg, Düsseldorf, Germany) operated at 32000*g* (25000rev./min.) and a flow rate of 10–12l./hr. They were then washed twice by resuspension in one-tenth the culture volume of water at 4°, followed by centrifugation for 30min. at 1750*g* (International model PR2, 850A head). After

washing, the cells were freeze-dried and stored over silica gel at 4°.

Reagents. All chemicals were of analytical-reagent grade except for tris buffer, which was British Drug Houses Ltd. (Poole, Dorset) laboratory-reagent grade.

Cytochrome c. This was prepared from ox-heart muscle by the method of Keilin & Hartree (1952) and contained 0·33% of iron.

METHODS

Spectrophotometry

Absorption spectra were obtained by means of a Cary model 14 recording spectrophotometer. A Zeiss model PMQ II spectrophotometer was also used for extinction determinations. Reduced preparations were obtained by addition of a slight excess of solid $Na_2S_2O_4$ to the pigment sample in buffer. Oxidized preparations were obtained by addition of a slight excess of 3mM-$K_3Fe(CN)_6$ to the pigment sample in buffer followed by dialysis against several changes of the buffer. When dilution was to be avoided, as in the determinations of the percentage reduction, a slight excess of finely powdered solid $K_3Fe(CN)_6$ was added. The percentage reduction of cytochrome 552 present in a partially reduced solution (as was required for the autoxidation experiments) was obtained by substitution in the equation:

$$\text{Reduction (\%)} = \frac{E^{1\,\text{cm.}}_{552} - E^{1\,\text{cm.}}_{552\,(\text{ox.})}}{E^{1\,\text{cm.}}_{552\,(\text{red.})} - E^{1\,\text{cm.}}_{552\,(\text{ox.})}} \times 100$$

where $E^{1\,\text{cm.}}_{552}$ is the extinction of the partially reduced solution, and the other extinction values were obtained from a sample of the solution that was completely oxidized and then completely reduced as above.

Electrophoresis

Starch-gel electrophoresis. This was carried out on horizontal gels with Connaught Laboratories (Toronto, Canada) hydrolysed starch made up in 0·023M-sodium borate buffer, pH 8·8, as described by Smithies (1955). Reduced cytochrome 552 and ox-heart cytochrome *c* were prepared by the addition of a slight excess of $Na_2S_2O_4$ followed by dialysis for 5hr. against several changes of the above borate buffer. Oxidized cytochromes were prepared by addition of a slight excess of $K_3Fe(CN)_6$ followed by dialysis as for the reduced pigments. Cytochromoid *c*, as obtained below, was already fully oxidized; the reduced form was prepared by addition of a slight excess of $Na_2S_2O_4$ immediately before electrophoresis. Electrophoresis was carried out for about 1hr. at 4° and 6v/cm. Amido Black 10B was used for staining.

Moving-boundary electrophoresis. Mobility determinations were carried out with crystalline samples of reduced cytochrome 552 and oxidized cytochromoid *c* in buffers of *I* 0·1 in the 2ml. cell of a Perkin–Elmer model 38 Tiselius-type electrophoresis apparatus. Buffers were made up as described by Datta & Grzybowski (1961) and checked at the temperature of electrophoresis (1·5°) with a glass electrode. Solutions for electrophoresis were equilibrated by dialysis against the appropriate buffer. Electrophoresis was carried out for 3–4hr. at a potential gradient of about 10v/cm.

Assay methods

Amino acid analysis. A Beckman model 120B amino acid analyser was used. Samples were hydrolysed for 22hr. in 6N-HCl at 110° in sealed evacuated tubes. For cytochrome 552 the results are the means of two separate hydrolyses with duplicate determinations on each hydrolysate. Ammonia was estimated on one such hydrolysate by a Conway-type distillation followed by a modification of the colorimetric procedure of Brown, Duda, Korkes & Handler (1957). Owing to the small amount available, results with cytochromoid *c* are the means of duplicate determinations on one hydrolysate. The ammonia estimation was carried out in the amino acid analyser.

Iron analysis. The method of Drabkin (1941) was used with slight modification (Henderson & Rawlinson, 1956).

Isolation and purification of cytochrome 552 *and cytochromoid* c

'pH 7 *alumina*'. Alumina (Judex, chromatographic grade) was heated with N-NaOH (80°) for 20min., washed thoroughly with water, and the fraction settling in 3min. retained. The alkaline treatment and washing was then repeated. The alumina was next stirred with N-HCl for 20min. and then washed with water. The pH of the suspension was adjusted to 7 with N-NaOH and, after further washing with water, the alumina was equilibrated to pH 7·0 by repeated washing with 0·1M-sodium phosphate buffer, pH 7·0. Columns of the alumina were washed free from fines with 0·01M-sodium phosphate buffer, pH 7·0; this was checked by ensuring that $E^{1\,\text{cm.}}_{280}$ of the effluent was not more than 0·003. Immediately before application of the sample, the column was washed with three times the alumina-volume of water.

'pH 7·5 *resin*'. Amberlite CG-50 (100–200 mesh) was stirred with 5% (w/v) KOH at 60° for 20min., then thoroughly washed with water, and the fraction settling in 15sec.–1min. retained. This treatment was repeated and the resin was then stirred with 5% (v/v) H_2SO_4 at 60° for 10min. After washing with water, the pH of the suspension was adjusted to 7·5 with 5% (w/v) KOH, after which it was washed with water. To achieve complete equilibration to pH 7·5 it was necessary to wash repeatedly with 0·1M-potassium phosphate buffer, pH 7·5. Columns of this resin were tested for fines and washed with water immediately before application of the sample, and in a manner similar to that for the 'pH 7 alumina' columns.

'pH 8·0 *resin*'. The column was poured with 'pH 7·5 resin', then 2M-ammonium phosphate buffer, pH 8·0, was passed through the column: at least 50ml. for a 10cm. × 1cm. column. The column was then washed with water and the effluent checked for fines and proteins as described above.

Disruption of cells and extraction of pigments. Disruption of the cells was carried out with an MSE 500w, 20 kcyc./sec. ultrasonic generator. Freeze-dried cells (25g.) were suspended in 250ml. of 0·1M-tris buffer, pH 8·0, and the suspension was separated into two approximately equal portions. Disruption of one portion was carried out for 1min. in an ice bath; then, while this was being stirred in an ice bath, the second portion was similarly disrupted for 1min. This procedure was maintained for a disruption time of 30min. for each portion. The resultant mixture

was diluted with 100ml. of tris buffer, pH 8·0, and extracted overnight with continuous stirring. It was then sedimented for 30min. at 17500*g* (Servall SS1). The supernatant was separated and stored at 4°, and the precipitate was resuspended in 160ml. of tris buffer, pH 8·0, and disrupted in a similar manner to that described above for a further total disruption time of 60min. The mixture was again diluted with 100ml. of tris buffer, pH 8·0, and extracted overnight with continuous stirring. The residue was then removed by centrifugation for 30min. at 17500*g*. The supernatants from the first and second extractions were combined and dialysed against 15vol. of water with four changes over 2 days. Fine particles, which formed during dialysis, were removed by centrifugation at 10000*g* for 20min. to avoid blocking the column at the next stage. In Fig. 1 the release of haemoprotein is compared with time of ultrasonic treatment at pH 8·0.

Separation and crystallization of pigments. The dialysed solution was first chromatographed on a column (17cm. × 2·5cm.) of 'pH 7 alumina'. Cytochrome 552 and cytochromoid *c* were adsorbed at the top of the column whereas the bacteriochlorophyll and carotenoids passed through. After being well washed with water, the haemoproteins were eluted with 120ml. of 2M-ammonium phosphate buffer, pH 8·0, and the eluate was dialysed against 10vol. of water with four changes over 2 days. A colourless precipitate that formed during dialysis was removed by centrifugation. The impure mixture of cytochrome 552 and cytochromoid *c* was next applied to a column (17cm. × 1·5cm.) of 'pH 7·5 resin'; this left the two pigments adsorbed at the top of the column, which was then washed with water. The washing removed some yellow–brown material, which may have been modified haemoprotein, as it showed a very weak haemochromogen-type spectrum on reduction with $Na_2S_2O_4$. Continued washing resulted in a separation of cytochromoid *c* from cytochrome 552. The oxidized cytochrome 552 was strongly adsorbed as a red zone at the top of the column with a coloured pink front of the less strongly adsorbed reduced form, whereas the cytochromoid *c* separated as a slowly moving brown zone becoming more diffuse as it moved down the column. When it was apparent that most of the cytochromoid *c* had been separated from the cytochrome 552 and while the former was still on the column, the resin that contained the cytochrome 552 was removed by suction as a slurry with water and poured on top of another small column (2cm. × 1cm.) of 'pH 7·5 resin'. Cytochrome 552 and cytochromoid *c* were then eluted from their separate columns with 30ml. of 0·1M-potassium phosphate buffer, pH 7·5, and the eluates were dialysed against water.

Cytochromoid *c* was further purified by passage through a column (10cm. × 1cm.) of 'pH 8·0 resin', which strongly

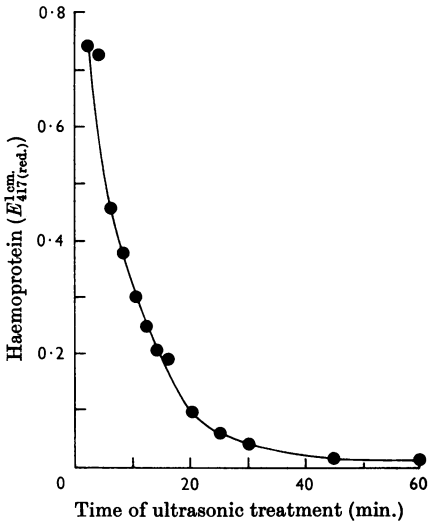

Fig. 1. Plot of release of haemoprotein from *Rps. palustris* against time of ultrasonic treatment. A suspension of 3g. dry wt. of cells was made in 40ml. of 0·1M-tris buffer, pH 8·0; this was kept in an ice bath throughout the ultrasonic treatment. Disruption was carried out for 1min. periods alternating with 1min. cooling. At each successive point shown, the suspension was centrifuged at 17500*g* for 15min. and the supernatant was removed and brought to 20% (w/v) by the addition of solid $(NH_4)_2SO_4$. This treatment precipitated the bacteriochlorophyll and carotenoids and left the haemoproteins in solution. The latter were separated by centrifugation (1500*g*) and estimated by measurement of $E^{1cm.}_{417}$ after reduction with $Na_2S_2O_4$.

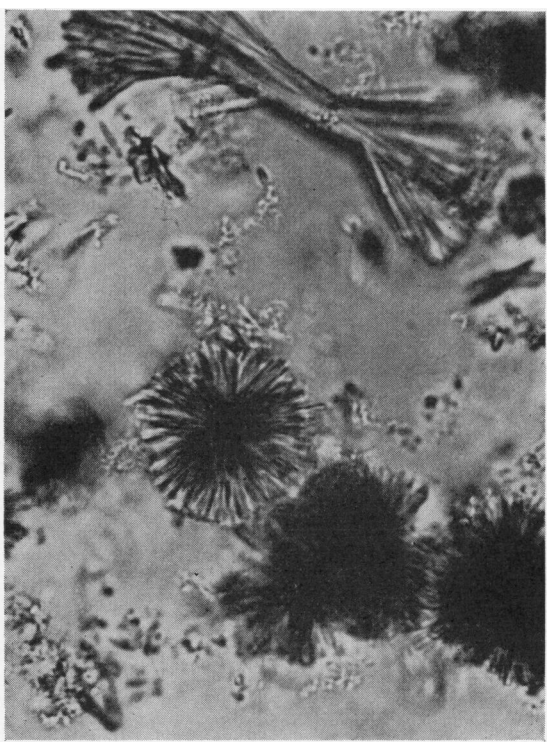

Fig. 2. Crystals of oxidized *Rps. palustris* cytochromoid *c*. Magnification × 725.

adsorbed any remaining cytochrome 552 whereas the cytochromoid *c* was not adsorbed. The cytochromoid *c* was then applied to a column (10 cm. × 1 cm.) of 'pH 7·5 resin', washed well with water and then eluted with 0·1 M-potassium phosphate buffer, pH 7·5. At this stage the following extinction ratios were obtained for the oxidized pigment: E_{642}/E_{283} 0·13 and E_{395}/E_{276} 3·61. Oxidized cytochromoid *c* was obtained in crystalline form by dialysing the above eluate against saturated $(NH_4)_2SO_4$ for 24 hr. (Fig. 2).

Further purification of cytochrome 552 was carried out by adsorption on a column (10 cm. × 1 cm.) of 'pH 8·0 resin', after which it was washed with water to remove any remaining cytochromoid *c*. The cytochrome 552 that was then eluted with 0·1 M-potassium phosphate buffer, pH 7·5, possessed an extinction ratio $E_{552\,(red.)}/E_{275\,(ox.)}$ 1·12. The preparation was obtained in crystalline form at this stage by dialysis of the reduced pigment against 70% saturated $(NH_4)_2SO_4$. The first crystals appeared after 48 hr. as rosettes of red needles; after 8 weeks the rosettes were largely replaced by lanceolate forms and some rectangular plates.

RESULTS

Spectrophotometry. Absorption spectra of both oxidized and reduced forms of cytochrome 552 and cytochromoid *c* were obtained in the visible and ultraviolet regions of the spectrum from solutions in 0·05 M-potassium phosphate buffer, pH 7·0. For cytochromoid *c* the dissociation of the carbon monoxide complex is significant at atmospheric pressure (Kamen, 1963*a*; Bartsch, 1963). Therefore it was prepared in a cuvette equipped with a gassing attachment that permitted the sample to be first flushed with carbon monoxide, and then the pressure raised to 1 atm. above atmospheric pressure. While under this increased pressure, a slight excess of sodium dithionite was introduced from a side arm and the sample examined in the spectrophotometer. The spectra obtained indicated no significant difference from those published by Bartsch (1963) and de Klerk *et al.* (1965). Extinction ratios of the absorption maxima obtained from these spectra are presented in Table 1.

On an iron-content basis, $\epsilon_{552\,(red.)}$ is $23{\cdot}2 \times 10^3$ l. mole^{-1} cm.$^{-1}$ for cytochrome 552 at pH 7·0 in 0·05 M-potassium phosphate buffer and this value was used for estimations of concentration. Insufficient cytochromoid *c* was available for determination of ϵ, so in this case concentrations were estimated by using the value of $\epsilon_{435\,(red.)}$, 184×10^3 l. mole^{-1} cm.$^{-1}$, given by Bartsch (1963).

Starch-gel electrophoresis. The results of starch-gel electrophoresis in 0·023 M-sodium borate buffer, pH 8·8, of the oxidized and reduced forms of *Rps. palustris* cytochrome 552 and cytochromoid *c* and of ox-heart cytochrome *c* are compared in Fig. 3. Oxidized and reduced cytochrome 552 showed comparable rates of migration towards the cathode whereas reduced ox-heart cytochrome *c* migrated more rapidly towards the cathode than its oxidized form. The oxidized form of cytochromoid *c*

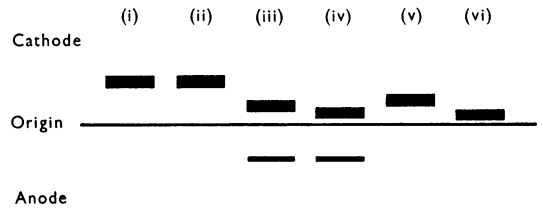

Fig. 3. Starch-gel electrophoresis of *Rps. palustris* cytochrome 552 and cytochromoid *c* and ox-heart cytochrome *c* in borate buffer, pH 8·8. (i) Oxidized cytochrome 552; (ii) reduced cytochrome 552; (iii) reduced ox-heart cytochrome *c*; (iv) oxidized ox-heart cytochrome *c*; (v) oxidized cytochromoid *c*; (vi) reduced cytochromoid *c*. Impurities in the ox-heart cytochrome *c* preparation which migrated to the anode are indicated in (iii) and (iv). Electrophoresis was carried out for 45 min. at 4° and 6 v/cm.; Amido Black 10B was used for staining.

Table 1. *Extinction ratios of crystalline preparations of* Rps. palustris *cytochrome* 552 *and cytochromoid* c

Cytochrome 552		Cytochromoid *c*			
Wavelength (x mμ)	$\frac{E_x}{E_{527\,(ox.)}}$	Wavelength (x' mμ)	$\frac{E_{x'}}{E_{552\,(red.)}}$	Wavelength (x'' mμ)	$\frac{E_{x''}}{E_{570\,(CO-red.)}}$
Oxidized		Oxidized		CO–reduced	
527	1·00	642	0·277	570	1·00
412	10·5	500	0·98	535	1·19
275	2·20	398	7·88	418	22·2
		283	2·17		
Reduced		Reduced			
552	2·47	552	1·00		
522	1·59	426	8·75		
418	12·8				

migrated more rapidly towards the cathode than the reduced form. Cytochrome 552 migrated more rapidly than reduced ox-heart cytochrome *c*, which migrated at a slightly lower rate than oxidized cytochromoid *c*. Relatively short runs were essential, owing to the tendencies for oxidized ox-heart cytochrome *c* to become reduced during electrophoresis, and for reduced cytochromoid *c* to become oxidized. In each case the respective change in redox state was followed by an increase in the rate of migration of the pigment. Impurities in the ox-heart cytochrome *c*, which are known to migrate towards the anode under these conditions (Henderson & Paléus, 1963), are indicated in Fig. 3.

Moving-boundary electrophoresis. Electrophoretic mobilities of cytochrome 552 in the pH range 4·0–11·1 in buffers of *I* 0·1 are plotted against pH in Fig. 4. The isoelectric point of 10·6 for cytochrome 552 is close to the value 10·65 obtained by Theorell & Åkesson (1941) for ox-heart cytochrome *c*.

Electrophoretic mobilities of cytochromoid *c* in the pH range 5·0–11·1 in buffers of *I* 0·1 are plotted against pH in Fig. 5. As insufficient pigment was available to enable a fresh sample to be taken for each run, each sample was used as far as possible for not more than two runs and then always first at a near-neutral pH value followed by a more alkaline or acid value. Nevertheless, difficulty was experienced in obtaining consistent mobility results at pH 7–8. At extremes of the pH range investigated, these difficulties were not experienced and the mobilities fitted into the expected pattern. Disregarding the equivocal results referred to, the remaining mobilities fit a curve that shows zero mobility at pH 9·7.

Iron contents. A sample of cytochrome 552 with an extinction ratio $E_{552\,(red.)}/E_{275\,(ox.)}$ 1·12 contained 0·33% of iron. This means that the minimum molecular weight is about 17000. Insufficient cytochromoid *c* was available for assay of iron.

Amino acid compositions. Amino acid analyses of crystalline samples of cytochrome 552 and cytochromoid *c* are presented in Table 2. After hydrolysis there was some dark humin present in each case. When buffer was added to the hydrolysate after drying over potassium hydroxide, the humin was present as a finely divided suspension. After thorough stirring, this material was removed by centrifugation at 1500*g* and samples of the supernatant were taken for column-chromatographic analysis. Decomposition of serine and threonine by 10% (Smith & Stockell, 1954) was allowed for in the results. The cysteine residues covalently bound to the haem are incompletely disrupted under the conditions of hydrolysis (Theorell, 1938) and therefore the values obtained for half-cystine are very probably low. The amino acid compositions of cytochrome 552 and cytochromoid *c* reported are similar to those published by Kamen (1963*b*).

Autoxidation of cytochrome 552 and cytochromoid c. A 30 μM solution of cytochrome 552 in 0·05M-potassium phosphate buffer, pH 7·0, was partially

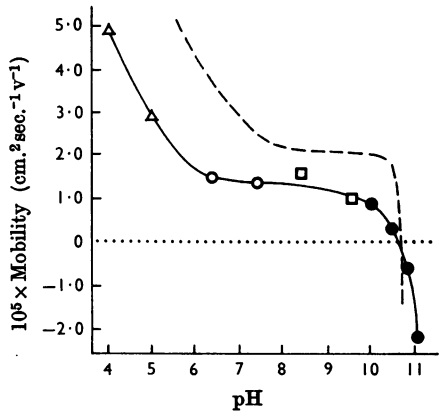

Fig. 4. Plot of electrophoretic mobilities of *Rps. palustris* cytochrome 552 against pH. The buffers, *I* 0·1, were sodium acetate (△), sodium–potassium phosphate (○), tris (□) and glycine (●). All buffers were prepared as described by Datta & Grzybowski (1961). Electrophoresis was carried out for 2–5 hr. at 1·5° and about 10 v/cm. ——, present results; – – – –, curve obtained by Theorell & Åkesson (1941) for ox-heart cytochrome *c*. The plotted mobilities are means from each limb of the cell.

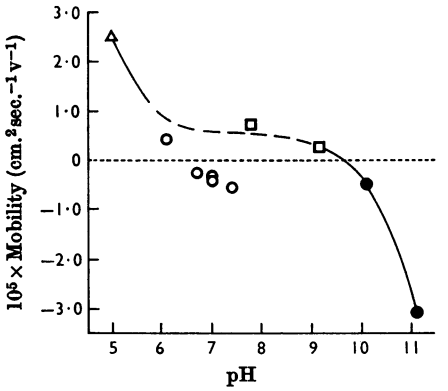

Fig. 5. Plot of electrophoretic mobilities of *Rps. palustris* cytochromoid *c* against pH. The buffers, *I* 0·1, were sodium acetate (△), sodium–potassium phosphate (○), tris (□) and glycine (●). All buffers were prepared as described by Datta & Grzybowski (1961). Electrophoresis was carried out for 1–5 hr. at 1·5° and about 10 v/cm. The plotted mobilities are means from each limb of the cell.

Table 2. *Amino acid analyses of* Rps. palustris *cytochrome* 552 *and cytochromoid* c *and horse-heart cytochrome* c

The cytochromoid *c* results are the means of duplicate determinations on a sample possessing the ratio $E_{642\,(ox.)}/E_{283\,(ox.)}$ 0·10. The cytochrome 552 results are the means of duplicate determinations on two samples each possessing the ratio $E_{552\,(red.)}/E_{275\,(ox.)}$ 1·12. Values have been corrected for 10% destruction during hydrolysis of serine and threonine (Smith & Stockell, 1954).

	Amino acid composition (moles/100 moles)		
	Rps. palustris cytochromoid *c*	*Rps. palustris* cytochrome 552	Horse-heart cytochrome *c* (from Margoliash, Kimmel, Hill & Schmidt, 1962)
Lys	12·1	10·4	18·3
His	0·8	1·5	2·9
NH_3	7·4	17	7·7
Arg	2·1	2·8	1·9
Asp	10·9	12·9	7·7
Thr	5·3	7·3	9·6
Ser	4·4	3·1	0
Glu	9·7	8·0	11·5
Pro	3·7	3·6	3·8
Gly	7·6	9·5	11·5
Ala	17·7	14·6	5·8
CyS (half)	0·9	0·8	1·9
Val	4·3	6·6	2·9
Met	2·2	2·4	1·9
Ile	5·4	1·7	5·8
Leu	8·2	8·0	5·8
Tyr	1·0	2·6	3·8
Phe	3·8	3·9	3·8
Trp	—	—	1·0

reduced with sodium dithionite and then dialysed against three changes of the above buffer. After this treatment the cytochrome was 70% reduced. It was then exposed to air in a test tube and shaken at intervals of several minutes at 24°. Over a period of 10hr. under these conditions, followed by a further 14hr. standing without shaking, there was no detectable change in extinction at 552mμ.

Solutions of 6μM-cytochromoid *c* in 0·05M-potassium phosphate buffer, pH 7·0, were placed in test tubes and reduced with a slight excess of sodium dithionite. The tubes were shaken at intervals of 30sec. and the decrease in extinction at 440mμ was observed. Samples from two different preparations, each possessing the ratio $E_{642\,(ox.)}/E_{283\,(ox.)}$ 0·13 at pH 7·0, were completely oxidized within 5min. Further samples from the above preparations were subjected to alkaline treatments as follows: (i) dialysed against 4mN-ammonia for 6hr. at 4° followed by equilibration against 0·05M-potassium phosphate buffer, pH 7·0; (ii) treated for 15hr. in 0·1M-potassium phosphate buffer, pH 11·6, at 4° followed by neutralization and equilibration against 0·05M-potassium phosphate buffer, pH 7·0. Each of the alkali-treated samples required 20min. for complete oxidation under the conditions used for the untreated samples.

DISCUSSION

The electrophoretic mobility–pH curves for both cytochromoid *c* and cytochrome 552 exhibit some degree of similarity to each other and to that for ox-heart cytochrome *c*. It is noteworthy that the isoelectric points of the last two named are so close despite a disparity in the ratios of their basic to acidic amino acids. It is probable that this disparity is offset by the relatively higher ammonia content of cytochrome 552. Although for reliable ammonia determinations a series of hydrolyses under varying conditions would be required, the results indicate that most of the carboxylic acid side chains of cytochrome 552 are present as amides. Under these conditions the ε-amino group of lysine (p*K* 10·5) probably determines the net charge on the molecule at alkaline pH values. These results disagree with the isoelectric point of pH 7·7 reported by Morita (1960). Mobilities were obtained by him at only three pH values, namely 7·0, 7·5 and 8·0, so that only one determination was carried out on the alkaline side of the reported isoelectric point. Neither the actual mobility values obtained, nor the strain of *Rps. palustris* used, was given. In the absence of further evidence it can only be assumed that Morita (1960) obtained a cytochrome 552 having different properties from the above, perhaps from a mutant strain.

The lower electrophoretic mobilities of cytochromoid *c* compared with those of cytochrome 552 and ox-heart cytochrome *c* at about pH 7 may be explained in part by the relatively low ammonia content of the cytochromoid *c*. When the basic and acidic amino acid composition of this pigment is taken into account, together with its ammonia content, only a small net positive charge is indicated in this pH region. If the charge under these conditions is such that protein–ion interaction or aggregation of the cytochromoid *c* occurs, then this may explain the variable mobility values observed at near-neutral pH values (see e.g. Longsworth, 1959). It was found during starch-gel electrophoresis that oxidized cytochromoid *c* migrated more rapidly than the reduced form. A difference in migration rate during zone electrophoresis, dependent to some extent on the state of oxidation of the pigment, has long been known for heart-muscle cytochrome *c* (Paléus, 1952). It is now well

established that the latter pigment may, according to its method of preparation, be present in solution in several reversibly interchangeable polymeric forms (Margoliash & Lustgarten, 1962). It seems likely therefore that the variations in mobilities observed during starch-gel electrophoresis, which were dependent on the redox state of the pigments, were due to the presence of polymers.

Our preparations of cytochromoid *c* were initially autoxidizable and became significantly less so on treatment with alkali. This lends support to the view that, at least for the pigment from the above strain, it is the unmodified form that is autoxidizable.

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